PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) **International Patent Classification** 6 :
A01N 1/02, C12Q 1/68, G01N 33/53, 33/566, 33/567, A61K 38/00, C07K 1/00, 5/00, 7/00, 14/00, 16/00, 17/00

A1

(11) **International Publication Number:** **WO 98/08379**

(43) **International Publication Date:** 5 March 1998 (05.03.98)

(21) **International Application Number:** PCT/US97/14153

(22) **International Filing Date:** 27 August 1997 (27.08.97)

(30) **Priority Data:**
08/703,825 27 August 1996 (27.08.96) US

(71) **Applicants:** DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US). THE CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US).

(72) **Inventors:** CANTOR, Harvey, Ira; 9 Edmunds Way, Belmont, MA 02178 (US). WEBER, Georg, Franz; 163 Bellingham Road, Chestnut Hill, MA 02167 (US). ASHKAR, Samy; Apartment 606, 12 Stoneholm Street, Boston, MA 02115 (US). GLIMCHER, Melvin, J.; Cabot Estate, Apartment C-402, 241 Perkins Street, Boston, MA 02130 (US).

(74) **Agent:** MEIKLEJOHN, Anita, L.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) **Designated States:** CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) **Title:** METHODS FOR IDENTIFYING MODULATORS OF CELLULAR INTERACTIONS MEDIATED BY OSTEOPONTIN

(57) **Abstract**

The invention features a method for determining whether an agent modulates binding of CD44 to osteopontin. This method includes: (a) providing an osteopontin polypeptide; (b) exposing the osteopontin polypeptide to a CD44 polypeptide in the presence of the agent; (c) measuring the binding of the osteopontin polypeptide to the CD44 polypeptide; whereby, binding of the osteopontin polypeptide to the CD44 polypeptide in the presence of the agent which differs from the binding of the osteopontin poylpeptide to the CD44 polypeptide in the absence of the agent indicates that the agent modulates the binding of CD44 to osteopontin. The invention also includes other methods for identifying agents which modulate osteopontin-mediated binding, adhesion, chemotaxis, or inhibition of apoptosis. The invention also features osteopontin polypeptides which bind to CD44.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHODS FOR IDENTIFYING MODULATORS OF CELLULAR INTERACTIONS MEDIATED BY OSTEOPONTIN

Background of the Invention

Osteopontin, also referred to as Eta1, is a phosphoprotein that is expressed soon after nonspecific activation of lymphocytes, including T cells and certain natural killer (NK) cells. It is thought to play a role in recruiting and stimulating macrophages and lymphocytes as a non-specific response to microbial infection. It has been suggested that the ability to produce osteopontin contributes to the metastatic potential of cells. In addition, osteopontin is one of the major non-collagenous proteins in bone. It is secreted by osteoblasts, osteoclasts, and osteocytes and is thought to be important in both the mineralization and resorption of bone.

Summary of the Invention

In one aspect the invention features a method for determining whether an agent modulates binding of CD44 to osteopontin. This method includes:

(a) providing an osteopontin polypeptide;

(b) exposing the osteopontin polypeptide to a CD44 polypeptide in the presence of the agent; and

(c) measuring the binding of the osteopontin polypeptide to the CD44 polypeptide;

whereby, binding of the osteopontin polypeptide to the CD44 polypeptide in the presence of the agent which differs from the binding of the osteopontin polypeptide to the CD44 polypeptide in the absence of the agent indicates that the agent modulates the binding of CD44 to osteopontin.

In another aspect the invention features a method for determining whether an agent modulates binding of

osteopontin to CD44 receptor bearing cells. The method includes:

(a) providing an osteopontin polypeptide;

(b) exposing the osteopontin polypeptide to a CD44 receptor bearing cell in the presence of the agent; and

(c) measuring the binding of the osteopontin polypeptide to the CD44 receptor bearing cell;

whereby, binding of the osteopontin polypeptide to the CD44 receptor bearing cell in the presence of the agent which differs from the binding of the osteopontin polypeptide to the CD44 receptor bearing cell in the absence of the agent indicates that the agent modulates the binding of osteopontin to CD44 receptor bearing cells.

In yet another aspect, the invention features a method for determining whether an agent modulates adhesion of CD44 receptor bearing cells to osteopontin, including:

(a) providing an osteopontin polypeptide;

(b) exposing CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent; and

(c) measuring the adhesion of the CD44 receptor bearing cell to the osteopontin polypeptide;

whereby, adhesion of the CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent which differs from the adhesion of the CD44 receptor bearing cells to the osteopontin polypeptide in the absence of the agent indicates that the agent modulates the adhesion of CD44 receptor bearing cells to osteopontin.

The invention also features a method for determining whether an agent modulates osteopontin-mediated chemotaxis of CD44 receptor bearing cells, including:

(a) providing an osteopontin polypeptide;

(c) measuring the chemotactic response of CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent;

whereby, chemotactic response of the CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent which differs from the chemotactic response of the CD44 receptor bearing cells to the osteopontin polypeptide in the absence of the agent indicates that the agent modulates the osteopontin-mediated chemotactic response of CD44 receptor bearing cells.

In still another aspect, the invention features a method for determining whether an agent interferes with adhesion of CD44 receptor bearing cells to osteopontin, including:

(a) providing an osteopontin polypeptide;

(b) exposing CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent;

(c) measuring the adhesion of the CD44 receptor bearing cell to the osteopontin polypeptide;

whereby, adhesion of the CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent which is less than the adhesion of the CD44 receptor bearing cells to the osteopontin polypeptide in the absence of the agent indicates that the agent interferes with the adhesion of CD44 receptor bearing cells to osteopontin.

In various preferred embodiments the osteopontin polypeptide includes a polypeptide having the sequence of SEQ ID NO: 1; the osteopontin polypeptide is mature human osteopontin; and the CD44 receptor bearing cell is a metastatic tumor cell.

Another aspect of the invention features a method for determining whether an agent interferes with osteopontin-mediated cell migration, including:

(a) providing an osteopontin polypeptide; and

(b) measuring the chemotactic response of CD44 receptor bearing cells to the osteopontin polypeptide in the presence of the agent;

whereby, chemotaxis of the CD44 receptor bearing cells towards the osteopontin polypeptide in the presence of the agent that is less than the chemotaxis of the CD44 receptor bearing cells towards the osteopontin polypeptide in the absence of the agent indicates that the agent interferes with osteopontin-mediated cell migration. In various preferred embodiments of this method the osteopontin polypeptide includes a polypeptide having the sequence of SEQ ID NO: 1; the osteopontin polypeptide is mature human osteopontin; and the CD44 receptor bearing cell is a metastatic tumor cell.

The invention also features a method for evaluating whether an agent interferes with osteopontin-mediated inhibition of apoptosis, including:

a) treating cells so as to initiate apoptosis;

b) before or after the treatment, exposing the cells to the agent; and

c) exposing the treated and agent exposed cells to an osteopontin polypeptide;

d) measuring the viability of the treated and agent exposed cells subsequent to exposure to the osteopontin polypeptide;

whereby, cell viability of the treated and agent exposed cells subsequent to exposure to the osteopontin polypeptide that is less than the cell viability of treated cells not exposed to the agent, but exposed to the osteopontin polypeptide indicates that the agent interferes with osteopontin-mediated inhibition of apoptosis.

The invention also features a method for determining whether an agent interferes with binding of osteopontin to CD44 receptor bearing cells, including:

(a) providing an osteopontin polypeptide;

(b) exposing the osteopontin polypeptide to CD44 receptor bearing cells in the presence of the agent; and

(c) measuring the binding of the osteopontin polypeptide to the CD44 receptor bearing cells;

whereby, binding of the osteopontin polypeptide to the CD44 receptor bearing cells in the presence of the agent that is less than the binding of the osteopontin polypeptide to the CD44 receptor bearing cells in the absence of the agent indicates that the agent interferes with binding of osteopontin to CD44 receptor bearing cells.

In another aspect the invention features an isolated osteopontin polypeptide of fewer than 100 amino acid which binds to CD44. In a preferred embodiment the polypeptide includes the osteopontin polypeptide of SEQ ID NO: 1.

In another aspect the invention features the polypeptide of SEQ ID NO: 1. In another aspect the invention features polypeptides which are substantially identical to the polypeptide of SEQ ID NO: 1.

In another aspect, the invention features an isolated polypeptide fragment of the polypeptide of SEQ ID NO: 1 which includes at least 10 amino acids, preferably at least 15, 25, or even 50 amino acids, and which selectively binds to CD44 receptor. In yet another aspect, the invention feature polypeptides which are substantially identical to an isolated polypeptide fragment of the polypeptide of SEQ ID NO: 1 which includes at least 10 amino acids, preferably at least 15, 25, or even 50 amino acids. Preferably such polypeptides

selectively bind CD44 and/or modulate binding of osteopontin to CD44.

In yet another aspect, the invention features an isolated polypeptide fragment of CD44 having osteopontin binding activity. In preferred embodiments the CD44 polypeptide has between 100 and 10 amino acids, between 75 and 10 amino acids, between 50 and 10 amino acids, and between 25 and 10 amino acids. In another aspect, the invention features a polypeptide substantially identical to one of the aforementioned CD44 polypeptides. Preferably the polypeptide selectively binds osteopontin and/or interferes with the binding of CD44 to osteopontin.

In many cases it will be desirable to use side-by-side assays, one in the presence of the selected agent and one in the absence of the selected agent, determine the effect of an agent on binding, adhesion, chemotactic response, or inhibition of apoptosis. The assay in the absence of the selected agent is a control experiment conduct in the same manner and under the same conditions (number of cells, type of cell, concentration of protein, type of buffer, etc.) as the assay in the presence of the selected agent expect, of course, that the selected agent is not present. However, it is not always necessary or desirable to perform one control assay for each test assay experiment. In many cases a single control assay will be adequate for a number of test experiments. In some cases it will be possible to establish a baseline measurement from a number of control assays. This baseline can be used for comparison to test assays.

The methods of the invention can be used to identify agents which modulate (interfere with or enhance), interfere with, or enhance the binding, adhesion, or chemotactic response. Agents which interfere with the response being assayed decrease

binding, adhesion, chemotactic response, or inhibition of apoptosis compared to the response in the absence of the agent. Agents which enhance the response being assayed increase binding, adhesion, chemotactic response, or inhibition of apoptosis compared to the response in the absence of the agent.

All of the methods of the invention can employ a particular CD44 variant and osteopontin variant. For a given assay, the comparison between binding, adhesion, chemotactic response, or inhibition of apoptosis should employ the same CD44 variant and the same osteopontin variant. In preferred embodiments, the osteopontin polypeptide is an isolated osteopontin polypeptide.

There are numerous CD44 variants, many of which are expressed only by metastatic cells. Thus, the method of the invention can employ a tumor cell or metastatic as the CD44 receptor bearing cell. Alternatively, recombinantly produced cells expressing a selected CD44 variant can be used in the methods of the invention. In many cases it is desirable to use a cell which does not express the selected CD44 variant as a control to insure that the effect of an agent on Osteopontin binding to CD44 receptor bearing cells is truly CD44 receptor dependent. In some cases it will be desirable to identify agents which have little or no significant effect on the binding (or lack thereof) of osteopontin to cells which do not express a CD44 receptor (or express a different CD44 receptor). It may also be desirable to identify agents which interfere with the adhesion of cells expressing one or another of several different CD44 receptors, but do not interfere with the adhesion of cells bearing other CD44 receptors.

Agents which interfere with the binding of CD44 receptor bearing cells to Osteopontin can be identified using any method which permits determination of cell

binding. In one such assay Osteopontin (or a portion thereof which includes a CD44 receptor interaction domain) is immobilized, e.g, on a solid support, and CD44 receptor expressing cells are exposed to the immobilized Osteopontin. The cells are allowed to interact with the immobilized Osteopontin in the presence (and the absence of a selected agent). Non-adherent cells are removed and adherent cells are counted. Alternatively, the binding of isolated, detectably labeled Osteopontin or Osteopontin polypeptides to CD44 receptor bearing cells can be measured using standard methods.

Various forms of osteopontin can be used in the method of the invention including: phosphorylated osteopontin and osteopontin that is both phosphorylated and glycosylated. Preferably, these post-translational modifications are identical to those present in a naturally-occurring form of osteopontin.

A "CD44 receptor bearing cell" is a cell which expresses a protein encoded by the CD44 gene. CD44-bearing cells include cells which express any of the various isoforms of CD44 generated by alternate RNA splicing a post-translational modification.

Osteopontin binding to CD44 receptor is binding that is specific, dose dependent, insensitive to competition by GRGDS peptide, inhibited by the presence of certain anti-CD44 antibodies, and sensitive to competition by hyaluronate.

The term "isolated osteopontin polypeptide" refers to full-length osteopontin (or a polypeptide fragment thereof; preferably of 15 amino acids, more preferably of 25 amino acids, even more preferably of at least 50 or even 65 or 75 amino acids) which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more

preferably at least 90%, and most preferably at least 99%, by weight, the selected polypeptide.

The term "isolated CD44 polypeptide" refers to full-length CD44 or a polypeptide fragment thereof; preferably of 15 amino acids, more preferably of 25 amino acids, even more preferably of at least 50 or even 65 or 75 or 100 amino acids) which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the selected polypeptide.

Any procedure known in the art can be used to identify agents which interfere with chemotaxis mediated by osteopontin and CD44. For example, one can observe migration in of cells across a gradient formed by osteopontin.

By "substantially identical" is meant a polypeptide or nucleic acid having a sequence that is at least 85%, preferably 90%, and more preferably 95% or more identical to the sequence of the reference amino acid or nucleic acid sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a specific percent identity to a reference polypeptide of a defined length, the percent identity is relative to the reference peptide. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

The experiments described below demonstrate that Osteopontin can induce CD44-dependent chemotaxis and that CD44-expressing cells can adhere to Osteopontin. These results suggest that the ability of Osteopontin to regulate inflammation, bone formation, and angiogenesis, which has been previously attributed to ligation of $\alpha_v\beta_3$ integrins, likely depends on an interaction with CD44. In inflammatory responses, antigen stimulation of lymphocytes leads to enhanced expression of a CD44 splice variant and secretion of Osteopontin. The results described below indicate that Osteopontin (but not HA) can induce CD44-dependent chemotaxis (Table 1), whereas HA (but not Osteopontin) induces CD44-dependent cell aggregation. The interaction between Osteopontin and

CD44 on activated lymphocytes and monocytes may mediate migration out of the bloodstream into sites of inflammation, where additional interactions between CD44 and HA may mobilize and activate these emigrant cells.

CD44 has also been implicated in tumor metastasis, although its precise role in this process has been unclear. Osteopontin, but not HA, can induce CD44-dependent chemotaxis, while HA, but not Osteopontin, induces CD44-dependent cell aggregation. Thus, it is possible that Osteopontin-CD44 interactions play a role in metastatic spread of tumor cells, while HA-CD44 interactions allow tumor cells to colonize new tissues.

The identification of agents that can interfere with the interaction between Osteopontin and CD44 may lead to new approaches intended to inhibit this mechanism of tumor invasion.

Agents interfere with the interaction between Osteopontin and CD44 receptor will be useful in treatment of cancer because they have the potential to block tumor metastasis and spread. Moreover, because Osteopontin induces chemotaxis by endothelial cells leading to neovascularization, agents which interfere with Osteopontin may reduce neovascularization of tumors. Further, because interactions between CD44 receptor and osteopontin may be important for immune cell migration, agents which interfere with the interaction between CD44 receptor and osteopontin may be useful in the treatment of autoimmune diseases.

Brief Description of the Drawings

Figure 1A is a graph illustrating the results of experiments used to measure the binding of soluble osteopontin to cells which express a CD44 receptor (A31.C1 cells; open circles), mock transfected cells which do not express a CD44 receptor (A31.MLV cells; open squares), and a second type of mock transfected cell

which does not express a CD44 receptor (filled circles; A31.E6 cells).

Figure 1B is a graph illustrating the results of experiments used to measure the binding of soluble BSA to cells which express a CD44 receptor (A31.C1 cells; open circles), mock transfected cells which do not express a CD44 receptor (A31.MLV cells; open squares), and a second type of mock transfected cell which does not express a CD44 receptor (filled circles; A31.E6 cells).

Figure 2 is a graph depicting the results of programmed cell death assays. Prevention of apoptosis by plate-bound phosphorylated Osteopontin was compared to other immobilized relevant compounds after starvation of NIH 3T3 T/2 cells in PBS containing 1.2 mM $CaCl_2$ and 1.0 mM $MgCl_2$ or after heat shock at 42°C for 30 min. After 48 hours, cells were harvested and analyzed for viability by Trypan blue exclusion.

Description of the Preferred Embodiments

Production of Osteopontin

Osteopontin (Opn) genes, proteins encoded by osteopontin genes, methods for producing recombinant osteopontin, methods for determining whether a given cell binds to osteopontin, are described in Cantor et al., U.S. Patent 5,049,659 and Cantor et al., U.S. Patent 5,238,839, both of which are hereby incorporated by reference (These patents refer to osteopontin by its earlier name Ap-1). In addition, Patarca et al. (*J. Exp. Med.* 170:145, 1989) and Oldberg et al. (*Proc. Nat'l Acad. Sci. USA* 83:8819, 1986) describe the identification of osteopontin. There are several variant forms of osteopontin described by Patarca et al. (*Crit. Rev. Immunol.* 13:225, 1993), Rodan, *Ann. N.Y. Acad. Sci.* 760:1, 1995) and Askar et al. (*Biochem. Biophys. Res. Comm.* 191:126, 1993).

Generation of Cells Expressing CD44

There are a large number of CD44 receptor variants generated, for the most part, by alternative splicing (Gunthert, *Current Topics in Microbiology and Immunology* 184:47, 1993). In many cases it is desirable to identify compounds which increase or decrease the binding of osteopontin to a particular CD44 variant. A number of CD44 variants and CD44 bearing cells are described in *The Lancet* 340:1053, 1992.

Sequences encoding CD44 variants from various cell types can be isolated by PCR amplification using a pair of primers designed to amplify all CD44 variants. For example, primers "Cr" (5'-CAGAATTCCTCGATCTCCTGGTAAGGAG-3') and "Cf" (5'-TAGGATCCTTGCCTCAACTGTGCACTCA-3') can be used to amplify sequences from a variety of cell lines. These primers were used to amplify cDNA from K8 cells (differentiated osteosarcoma cells that display high metastatic activity; Schmidt et al., *Differentiation* 39:151, 1988). The resulting DNA was cloned into the BamHI/EcoRI sites of the expression vector pRc/RSV and used to transform *E. Coli*.

Since this strategy amplifies and clones all CD44 variants present in the cDNA, specific clones coding for CD44v (exon 10 variants) (Ann. N.Y. were identified by colony blot hybridization using an exon 10 specific probe (CAGGGGAAGAACCCCCCTACCCCAAGTGAAGACTCC). Other specific CD44 variants can be identified in a similar manner using an appropriate, specific probe.

To create cells expressing CD44v, a clone encoding the CD44v variant was transfected into target cells and stable transfectants were isolated using standard procedures. In addition CD44v cDNA was inserted into eukaryotic expression vector pcDNA III/neo (Invitrogen; San Diego, CA). In this construct CD44v expression is under the control of enhancer/promoter sequences from the early gene of human cytomegalovirus. This construct also

includes the SV40 polyadenylation signal, the SV40 transcription termination signal, and, for selection of G418 resistant stable cell lines, the neomycin resistance gene. This CD44v expression construct was used to stably transfect the A31 mouse embryonic cell line by electroporation and selection by G418 resistance and adherence to HA to create cell line A31.C1.

This cell line, and other cell lines expressing CD44 variants, can be used in screening assays to identify compounds which increase or decrease the osteopontin/CD44 mediated effects.

Osteopontin Binding to CD44 Expressed on the Surface of Cells

Osteopontin binding to cellular CD44 was investigated using the CD44-expressing cells, A31.C1 cells, and mock-transfected cells, A31.MLV cells and A31.E6 cells, which do not display detectable CD44.

A31.C1, A31.MLV, and A31.E6 cells were incubated with various concentrations of biotinylated Osteopontin in 150 μl of calcium- and magnesium-free Phosphate Buffered Saline (to avoid binding to integrins) or 150 μl of biotinylated BSA followed by phycoerythrin-streptavidin. Briefly, 0.2 x 10^6 cells were incubated with biotinylated ligand in 150 μl of calcium- and magnesium-free phosphate-buffered saline (PBS) for 30 in at 37°C followed by fixation in 1% paraformaldehyde for 10 min on ice. After resuspension in calcium- and magnesium-free PBS, phycoerythrin-streptavidin was added at 1:100 dilution for 20 min on ice, and cells were washed and analyzed with a Coulter Profile flow cytometer.

The results of these binding studies are presented in Figure 1A and Figure 1B. The CD44-transfected A31.C1 cells bind Osteopontin in a dose-dependent manner, whereas mock-transfected A31.MLV cells or A31.E6 cells do

not display significant binding activity. Binding does not reflect nonspecific effects associated with CD44 expression because the transfectants do not show enhanced binding to biotinylated BSA compared with the results obtained with mock-transfected A31.MLV cells. At very high concentrations of 40 μg of BSA in 150 μl of solution, BSA still bound equally well to A31.C1 cells (13.58 fluorescence units) and A31.MLV cells (13.60 fluorescence units). Moreover, separate studies demonstrated that binding of biotin-labeled Osteopontin to A31.C1 CD44 transfectants was inhibited by an antibody to CD44 but not by an irrelevant antibody.

Acquisition of Osteopontin-binding activity after stable expression of CD44 and inhibition by antibodies to CD44 suggested a direct interaction between Osteopontin and CD44. This was confirmed by analyzing proteins that bound to recombinant glutathione-S-transferase (GST)-Osteopontin (which lacks glycosyl moieties, including HA) immobilized on Sepharose 4B (Ashkar et al., *Biochem. Biophys. Res. Comm.* 191:126, 1993). Coomassie-stained electrophoresis gels of the desalted eluate showed co-migration of the most prominent band with CD44 purified from the same cell line. Several bands of lower molecular weight were also noted, the number of which varied inversely with the stringency of the buffer conditions. The specificity of binding to immobilized Osteopontin was confirmed by comparison of separate eluates from a GST-Osteopontin column and a control GST column on SDS gel electrophoresis. The eluate from the GST-Osteopontin column, but not from the GST column, contained CD44 according to protein immunoblotting with mAb KM 81 (antibody to CD44), which revealed a single band that comigrated with a KM 81-reactive band from whole AF3.G7 T cell lysate and from affinity-purified CD44 on native polyacrylamide gel electrophoresis.

Depletion of CD44 from cell lysates with antibody removed this band from the material eluted from the GST-Osteopontin columns. Similar results were obtained for Osteopontin-binding material from WEHI-3B cells, K8 cells, and A31.C1 transfectants.

Biological Effects of Osteopontin Binding to CD44

Cellular adhesion participates in both cell aggregation and motility. To investigate the role of the interaction between Osteopontin and CD44, cell adhesion assays were performed as follows. Plates (96 well) were coated with either 10 μg/ml of Osteopontin, 10 μg/ml of fibronectin, or 100 μg/ml of HA for 18 hrs at 4°C followed by blocking with 1 mg/ml of BSA for 2 hours at room temperature. One thousand cells per well were incubated at 37°C in calcium- and magnesium-free PBS containing 100 μg/ml of BSA. After 30 min the cells were fixed in 4% paraformaldehyde in PBS and stained with toluidine blue. Attachment was assessed by counting the total number of cells per well. Inhibitors were added at the following concentrations: GRGDS peptide (1mM); Osteopontin (500 μg/ml), hyaluronic acid (HA; 1 mg/ml).

These studies revealed that A31.C1 cells, but not A31.MLV cells, adhered to plate-bound Osteopontin or HA in calcium- and magnesium-free medium, and binding to both immobilized ligands was inhibited by soluble Osteopontin or HA, but not by the GRGDS peptide (where G is Gly, R is Arg, D is Asp, and S is Ser). In contrast, both A31.C1 cells and A31.MLV cells adhered to fibronectin in calcium-containing cultures, and adherence was inhibited by the GRGDS peptide but not by HA or Osteopontin. The efficiency of adherence to Osteopontin and HA by A31.C1 cells was not affected by the presence or absence of calcium and magnesium. Treatment of A31.C1 cells with chondroitinase ABC did not inhibit specific binding or adherence of Osteopontin to CD44, whereas such

treatment eliminated HA-mediated aggregation and decreased attachment to HA by about 60%. These results suggest that unlike HA, Osteopontin binding to CD44 is not mediated by chondroitin sulfate.

Because Osteopontin can induce chemotaxis *in vivo*, we investigated whether this ligand and HA might cause CD44-dependent chemotaxis *in vitro*. The results of this study are presented in Table 1.

Migration of A31.C1 (CD44-expressing) cells and A31.MLV (mock-transfected) cells toward K8-derived Osteopontin was assessed by counting the numbers of cells that migrated across a polycarbonate filter after 4 hours (Wass et al., *J. Natl. Cancer Inst.* 66:927, 1981) in a modified Boyden chambers (Moses et al., Science 248:1408, 1990) . Briefly, surfaces of polycarbonate filters (pore size, 8 μm) were coated with 5 μg of fibronectin before 1 x 10 cells were added in 500 μl to the upper chamber and incubated at 37°C in the presence or absence of chemotactic agents in the lower chamber. After 4 hours the, the membranes were fixed in methanol and stained with hematoxylin and toluidine blue. Responding cells on the lower surface of the filter were counted microscopically and evaluated in triplicates.

Referring to Table 1, the results of Experiment 1 show the migration of CD44-transfected A31.C1 cells into lower chambers containing two concentrations of Osteopontin. The migration was reduced by increasing concentrations of Osteopontin in the upper chamber, which indicates that the response is chemotactic rather than chemokinetic. Still referring to Table 1, the result of Experiments 2 and 3 show the numbers of migrating A31.C1 cells (experiment 2) or A31.MLV cells (experiment 3) in chambers containing an Osteopontin gradient. The effects of including anti-Osteopontin or anti-CD44 in the upper or lower chambers are shown. Osteopontin-dependent

chemotaxis was noted for A31.C1 cells but not for A31.MLV cells. The migration of responding cells was inhibited by Osteopontin antibody (rabbit immunoglobulin, 1:200 dilution) (Ashkar et al., Ann. N.Y. Acad. Sci. 760:296, 1995) or anti-CD44 KM 81 (TIB 241 supernatant, 1:200 dilution). HA neither induced A31.C1 cell migration nor inhibited migration in response to Osteopontin. Still referring to Table 1, the results of Experiment 4 show that the chemotaxis of AF3.G7 cells toward recombinant Osteopontin is dose-dependent and inhibited by Osteopontin in the upper chamber. Hyaluronic acid did not exert chemotactic stimuli to AF3.G7 cells.

TABLE I

Lower chamber contents	Cell numbers with upper chamber contents of: Opn (ng) 0	20	50	100	200	Anti-Opn	Anti-CD44	HA (1 μg)
				Experiment 1: A31.C1 cells				
None	38±18	42±21	26±11	46±19	19±7	-	-	-
Opn (50 ng)	211±36**	174±48**	77±24**	65±12	13±4	-	-	-
Opn (100 ng)	276±46**	226±53**	196±40**	103±22**	87±34*	-	-	-
				Experiment 2: A31.C1 cells				
None	15±4	-	-	20±8	-	11±6	23±7	22±8
Opn (100 ng)	97±32**	-	-	32±13	-	67±22**	43±18	117±28**
Anti-Opn	11±4	-	-	9±5	-	18±7	23±10	28±11
Anti-CD44	17±6	-	-	34±11	-	26±10	9±4	19±9
HA (1 μg)	44±28	-	-	24±7	-	18±3	31±15	21±18
				Experiment 3: A31.MLV cells				
None	21±14	-	-	27±11	-	10±3	9±3	14±6
Opn (100 ng)	48±22	-	-	32±13	-	29±12	36±9	31±10
Anti-Opn	9±4	-	-	18±8	-	11±5	16±5	22±13
Anti-CD44	22±9	-	-	24±14	-	17±6	8±3	27±13
HA (1 μg)	24±8	-	-	31±16	-	21±8	22±7	19±4
				Experiment 4: AF3.G7 cells				
None	5±2	-	-	7±4	4±3	-	9±4	-
Opn (50 ng)	48±7*	-	-	21±8	11±6	-	15±7	-
Opn (100 ng)	33±11*	-	-	23±6	9±4	-	17±8	-
HA (100 μg)	6±3	-	-	5±2	8±3	-	2±1	-

*P < 0.05. **P < 0.01.

To summarize, CD44-expressing cells (A31.C1 cells) migrated toward Osteopontin in a manner reflecting chemotaxis rather than a nonspecific increase in motility (chemokinesis) (Table 1, Experiment 1) and were inhibited by antibodies to Osteopontin or CD44 (Table 1, Experiment 2). The mock-transfected cell line A31.MLV did not display chemotaxis to Osteopontin (Table 1, Experiment 3). In contrast to Osteopontin, the CD44 ligand HA did not induce significant chemotactic activity (Table 1, Experiments 2 and 3). The interaction between naturally expressed CD44 on AF3.G7 cells and Osteopontin also mediated a chemotactic response similar to that obtained after genetic transfer of CD44 into A31 cells (Table 1, experiment 4). $\alpha_v\beta_x$ integrins may also interact with Osteopontin, and $\alpha_v\beta_3$ mediates chemotaxis of smooth muscle cells. Although A31 and A31.C1 cells do not express integrin $\alpha_v\beta_3$, they may express small amounts of other α_v integrins. If so, these integrins do not contribute to the Osteopontin-dependent activation described here. CD44-negative A31.MLV cells do not bind, attach, or migrate toward Osteopontin, and the attachment and migration of CD44$^+$ A31.C1 cells is calcium-independent and inhibited by CD44 antibody but not by integrin β_3 antibody or GRGDS peptides.

HA mediates homotypic aggregation of CD44$^+$ hematopoietic cells and fibroblasts. A series of experiments confirmed that HA caused CD44$^+$ A31.C1 cells, but not A31.MLV cells, to aggregate and that aggregation was inhibited completely by antibody to CD44. In contrast, Osteopontin did not induce detectable aggregation at 10 to 100 times the concentrations necessary for attachment and chemotaxis. The failure of Osteopontin to mediate aggregation in these studies was not simply due to a small number of binding sites per molecule compared with HA because large aggregates of 5

to 10 Osteopontin molecules are formed at the concentrations used in these experiments.

Binding of Various Forms of Osteopontin to CD44 Receptor Bearing Cells

Various CD44-bearing cell lines as well as embryonic fibroblast A31 cells, which do not express CD44, were tested for specific binding to recombinant Osteopontin, which is neither phosphorylated nor glycosylated, recombinant Osteopontin that had been phosphorylated by Golgi kinases, and naturally-occuring Osteopontin purified from the osteosarcoma cell line K7. This last form of osteopontin is both phosphorylated and glycosylated. Binding was assayed by adding 2.5 pmol radiolabelled osteopontin (^{32}P or ^{125}I) to $1x10^6$ cells, incubating for 90 min at room temperature in the presence or absence of 200-fold excess of non-labeled osteopontin in a total volume of 250 μl PBS. The bound and unbound fractions were separated by centrifugation over dibutyl phthalate:dioctylphthalate (6:4) at 4°C. Radioactivity in pellets and supernatants was quantitated by liquid scintillation counting. Binding was considered to be specific if there was a substantial reduction in bound radioactivity after addition of non-labeled osteopontin. In each case the concentration of Osteopontin used was about half the saturating concentration for binding to Wehi3B cells. The results of this binding assay are presented in Table 2. All of the cell types in Table 2, except A.31 cells, express CD44.

TABLE 2: Differential Binding of Cells to Various Forms of Osteopontin

Cells	rOPN	rOPN-P	Phosphorylated Glycosylated OPN
Wehi 3B	-	+	+
AF3.G7	-	+	N.D.
P388	-	-	+
EL-4	N.D.	+	N.D.
L1210	N.D.	+	N.D.
OS mouse	N.D.	+	+
A31.C1	-	-	N.D.
A31	-	-	-

Identification of a CD44-binding Osteopontin Polypeptide

An osteopontin polypeptide which can mediate chemotaxis of CD44 expressing cells was identified by generating osteopontin polypeptides and testing their ability to promote chemotaxis.

A series of osteopontin polypeptides were generated by digesting purified osteopontin (approximately 0.5 mg) with trypsin (2% w/w) in 0.2 ml of 50 mM NH_4HCO_3, pH 8.0, for 20 hrs at 37°C. The products of the digestion reaction were treated with 50 µl of H_2O + 0.1% TFA. This digestion procedure results in the generation of a partial tryptic peptide library containing overlapping sets of peptides and eliminates the need to perform digestions with various proteases to ensure that all possible peptides are generated.

The tryptic products were resolved by HPLC chromatography on a C-18 column (25 cm x 0.46 cm). After injection of the reaction products, the column was washed for 10 min, eluted by linear gradient from 100% H_2O + 0.1% TFA to 60% CH_3CN + 0.55% TFA over 120 min, and then eluted with a second gradient from 60% CH_3CN to 80% CN_3CN over 30

min at a flow rate of 0.5 ml/min. The absorbance of the eluate at 219 nm was recorded continuously by an on-line chart recorder/integrator using a Gilson HM Holochrome detector, and 0.5 ml eluate fractions were collected. Fractions in each peak were pooled, lyophilized, and redissolved in water. The material from each peak was analyzed for the ability to stimulate chemotaxis of CD44-expressing cells and the ability to promote attachment of CD44-expressing cells as described below.

Osteopontin polypeptides were tested for their ability to act as a chemotactic ligand for CD44 using a standard Boyden chamber assay. The CD44-expressing cells used in this assay were murine fibroblast A31 cell transfectants that stably express CD44 (A31.C1 cells).

Briefly, surfaces of polycarbonate filters (pore size, 8 μm) were coated with 5 μg fibronectin before $1x10^3$ A31.C1 cells were added in 500 μl to the upper chamber of a modified Boyden chamber and incubated at 37°C in the presence or absence of a selected osteopontin polypeptide or other agent in the lower chamber. After 4 hours, the membranes were fixed in methanol and stained with hematoxilin/toluidine blue. Responding cells on the lower surface of the filter were counted and evaluated in triplicate.

The results of this study are summarized in Table 3 in which OPNCT refers to the C-terminal osteopontin thrombin cleavage product (amino acid 175 to the carboxy terminus), OPNNT refers to the N-terminal osteopontin thrombin cleavage product (amino acids 1- 174), and P65 refers to the osteopontin polypeptide having the following sequence: DSLAYGLRSK SRSFQVSDEQ YPDATDEDLT SHMKSGESKE SLDVIPVAQL LSMPSDQDNN GKGSH (SEQ ID NO: 1).

As can be seen from Table 3, osteopontin is chemotactic for A31.C1 cells and OPNNT is moderately chemotactic for A31.C1 cells. As can also be seen from

Table 3, OPNNT and P65 inhibit chemotaxis induced by osteopontin.

TABLE 3: Chemotaxis Mediated by Osteopontin and Osteopontin Polypeptides

		LOWER CHAMBER				
		PBS	**OPN**	**OPNCT**	**OPNNT**	**P65**
UPPER CHAMBER	**PBS**	42±8	231±34	389±52	69±9	92±21
	OPN	12±5	104±18	211±36	20±5	17±9
	OPNCT	16±3	64±10	167±26	19±3	13±3
	OPNNT	21±2	187±40	261±42	14±8	23±3
	P65	28±6	126±24	207±53	26±7	35±5

Osteopontin-mediated Adherence of CD44-bearing cells

The following experiments demonstrates that binding of osteopontin to CD44-expressing cells and adherence of CD44-bearing cells to osteopontin is independent of chondroitin sulfate.

Adherence of CD44-bearing cells to osteopontin was assessed as follows. Plates (96 well) were coated with either 10 μg/ml of osteopontin, 10 μg/ml fibronectin or 100 μg/ml HA for 18 hours at 4°C followed by blocking with 1 mg/ml BSA for 2 hours at room temperature. Approximately 1 x 10^6 CD44-bearing cells (A31.C1 cells), pre-treated with 0.1 unit of chondroitinase ABC (incubated for 30 min at 37°C in 0.1 unit chondroitinase ABC in 300 μl PBS) or left untreated, were added to each well in calcium- and magnesium-free PBS containing 100 μg/ml BSA and incubated at 37°C. After 30 min the cells were fixed in 4% paraformaldehyde in PBS and stained with toluidine blue. Attachment was assessed by counting the total number of cells per well. As a control, adherence

of chondroitinase treated and untreated A31.C1 cells to immobilized hyaluronate was measured.

Chondroitinase treatment of CD44-bearing cells did not significantly alter their adherence to immobilized osteopontin. In contrast, chondroitinase treatment sharply reduced the adherence of CD44-bearing cells to immobilized hyaluronate.

The effect of chondroitinase treatment on binding of osteopontin to CD44-bearing cells was assessed as follows. Briefly, $1x10^6$ A31.C1 cells were incubated with 0.1 unit of chondroitinase ABC in 300 μl PBS for 30 minutes at 37°C or were left untreated. The cells were washed and viability was confirmed by Trypan blue exclusion. The cells were then incubated with biotinylated osteopontin in 150 μl of calcium-free, magnesium-free PBS for 30 minutes at 37°C followed by fixation in 1% paraformaldehyde for 10 minutes on ice. After resuspension in calcium-free, magnesium-free PBS, PE-streptavidin was added at 1:100 dilution for 20 minutes on ice, cells were washed and analyzed using a Coulter Profile flow cytometer.

This experiment revealed that chondroitinase treatment does not significantly alter the binding of osteopontin to A31.C1 cells.

Osteopontin-Specific Antibodies

Synthetic peptides were conjugated to keyhole limpet hemocyanin using standard methods and polyclonal antibodies were raised in rabbits. Briefly, 0.5 ml of a 100 μg/ml peptide solution were injected and a blood sample was tested after four weeks for specific antibody activity. For the following three months booster injections of 25 μg in 500 μl were given every two weeks. During the next two months every four weeks. Finally, during the next six months one booster injection was given every three months. The rabbit was sacrificed and

bled after 1 year. Antibodies from the resulting plasma were purified by affinity chromatography on a Sepharose 4B column conjugated with the relevant peptide. The resulting purified antibody was then tested for its ability to inhibit the chemotaxis of CD44$^+$ cells to osteopontin using the techniques described above.

Osteopontin and *in vivo* migration of immune cells

Osteopontin may attract immune cells after subcutaneous or intraperitoneal injection. To test this mice can be injected with 0-20 μg Osteopontin intraperitoneally. After six hours a cell sample is obtained by peritoneal lavage. Red cells are lysed by dilution in hypotonic buffer and the total number of cells retrieved are counted followed by fluorescent antibody labeling with phycoerythrin-anti-CD44, combined with fluorescein conjugated anti-Mac-1, anti-B220, or anti-CD3 and dual color flow cytometric analysis. This assay can also be used to assess the effect of agents on Osteopontin-mediated cell migration.

Apoptosis

The interaction of Osteopontin and CD44 may prevent apoptosis. This was assessed by examining the effect of Osteopontin on starved NIH 3T3 T/2 cells in PBS containing 1.2 mM $CaCl_2$ and 1.0 mM $MgCl_2$ and NIH 3T3 T/2 cells exposed to heat shock treatment. After treatment cells were plated on recombinant Osteopontin, phosphorylated recombinant Osteopontin, polylysine, collagen, or fibronectin. After 24 to 48 hours, cells were harvested, stained with the benzimidine dye Hoechst 33342 and propidium iodide, and analyzed for apoptosis and necrosis by flow cytometry (Weber and Cantor, *Immunity* 2:363, 1995). The results of this study are presented in Figure 2. Phosphorylated recombinant Osteopontin, but not polylysine, collagen, fibronectin,

or non-phosphorylated Osteopontin, protected cells from death.

Normally, adherent cells undergo programmed cell death if attachment is prevented. Because attachment to Osteopontin appears to inhibit apoptosis, blocking the binding of cells to Osteopontin may induce apoptosis. This induction of apoptosis may be useful in treatment of cancer. Tumor cell death may be triggered by agents which interfere with the interaction between Osteopontin and a cell surface receptor, e.g., CD44. Because metastatic tumor cells express variants of CD44 that are not typically expressed by normal cells, such agents will be selective for tumor cells.

Agents Useful in Screening Methods

Agents for use in the screening assays of the invention can be obtained from any source. Libraries of synthetic and/or natural compounds are particularly useful. Numerous means are currently used for random and directed synthesis of saccharide, peptide, nucleic acid, and small molecule compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from Pan Laboratories (Bothell, WA) and MycoSearch (NC).

A number of methods can be used to create combinatorial libraries of compounds useful in high througput screening methods. A number of combinatorial approaches are discussed in Gordon et al., *J. Medicinal Chemistry* 37:1385, 1994 and Gallop et al., *J. Medicinal Chemistry* 37:1233, 1994.

Use

The present invention also encompasses pharmaceutical compositions which include: one or more agents identified using the above-described methods, one or more osteopontin polypeptides which modulate the binding of a CD44 polypeptide to a osteopontin polypetide, and/or one or more CD44 polypeptides which modulate the binding of a CD44 polypeptide to a osteopontin polypetide. These compositions include a pharmaceutically effective amount of the agent and/or polypeptide in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (Gennaro ed., 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH

buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes) may be utilized.

The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art based on generally accepted protocols for clinical studies.

In practicing the methods of the invention, the agents can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. An agent can be administered parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms.

What is claimed is:

1. A method for determining whether an agent modulates binding of CD44 to osteopontin, comprising:

(a) providing an osteopontin polypeptide;

(b) exposing said osteopontin polypeptide to a CD44 polypeptide in the presence of said agent; and

(c) measuring the binding of said osteopontin polypeptide to said CD44 polypeptide;

whereby, binding of said osteopontin polypeptide to said CD44 polypeptide in the presence of said agent which differs from the binding of said osteopontin polypeptide to said CD44 polypeptide in the absence of said agent indicates that said agent modulates the binding of CD44 to osteopontin.

2. A method for determining whether an agent modulates binding of osteopontin to CD44 receptor bearing cells, comprising:

(a) providing an osteopontin polypeptide;

(b) exposing said osteopontin polypeptide to a CD44 receptor bearing cell in the presence of said agent; and

(c) measuring the binding of said osteopontin polypeptide to said CD44 receptor bearing cell;

whereby, binding of said osteopontin polypeptide to said CD44 receptor bearing cell in the presence of said agent which differs from the binding of said osteopontin polypeptide to said CD44 receptor bearing cell in the absence of said agent indicates that said agent modulates the binding of osteopontin to CD44 receptor bearing cells.

3. A method for determining whether an agent modulates adhesion of CD44 receptor bearing cells to osteopontin, comprising:

(a) providing an osteopontin polypeptide;

(b) exposing CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent; and

(c) measuring the adhesion of said CD44 receptor bearing cell to said osteopontin polypeptide;

whereby, adhesion of said CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent which differs from the adhesion of said CD44 receptor bearing cells to said osteopontin polypeptide in the absence of said agent indicates that said agent modulates the adhesion of CD44 receptor bearing cells to osteopontin.

4. A method for determining whether an agent modulates osteopontin-mediated chemotaxis of CD44 receptor bearing cells, comprising:

(a) providing an osteopontin polypeptide; and

(c) measuring the chemotactic response of CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent;

whereby, chemotactic response of said CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent which differs from the chemotactic response of said CD44 receptor bearing cells to said osteopontin polypeptide in the absence of said agent indicates that said agent modulates the osteopontin-mediated chemotactic response of CD44 receptor bearing cells.

5. A method for determining whether an agent interferes with adhesion of CD44 receptor bearing cells to osteopontin, comprising:

(a) providing an osteopontin polypeptide;

(b) exposing CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent; and

(c) measuring the adhesion of said CD44 receptor bearing cell to said osteopontin polypeptide;

whereby, adhesion of said CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent which is less than the adhesion of said CD44 receptor bearing cells to said osteopontin polypeptide in the absence of said agent indicates that said agent interferes with the adhesion of CD44 receptor bearing cells to osteopontin.

6. The method of any of claims 1, 2, 3, 4, and 5 wherein said osteopontin polypeptide comprises a polypeptide having the sequence of SEQ ID NO: 1.

7. The method of any of claims 1, 2, 3, 4, and 5 wherein said osteopontin polypeptide is mature human osteopontin.

8. The method of claims 2, 3, 4, and 5 wherein said CD44 receptor bearing cell is a metastatic tumor cell.

9. A method for determining whether an agent interferes with osteopontin-mediated cell migration, comprising:

(a) providing an osteopontin polypeptide; and

(b) measuring the chemotactic response of CD44 receptor bearing cells to said osteopontin polypeptide in the presence of said agent;

whereby, chemotaxis of said CD44 receptor bearing cells towards said osteopontin polypeptide in the presence of said agent that is less than the chemotaxis

of said CD44 receptor bearing cells towards said osteopontin polypeptide in the absence of said agent indicates that said agent interferes with osteopontin-mediated cell migration.

10. The method of claim 9 wherein said osteopontin polypeptide comprises a polypeptide having the sequence of SEQ ID NO: 1.

11. The method of claim 9 wherein said osteopontin polypeptide is mature human osteopontin.

12. The method of claim 9 wherein said CD44 receptor bearing cell is a metastatic tumor cell.

13. A method for evaluating whether an agent interferes with osteopontin-mediated inhibition of apoptosis, comprising:

a) treating cells so as to initiate apoptosis;

b) before or after said treatment, exposing said cells to said agent;

c) exposing said treated and agent exposed cells to an osteopontin polypeptide; and

d) measuring the viability of said treated and agent exposed cells subsequent to exposure to said osteopontin polypeptide;

whereby, cell viability of said treated and agent exposed cells subsequent to exposure to said osteopontin polypetide that is less than the cell viability of treated cells not exposed to said agent, but exposed to said osteopontin polypeptide indicates that said agent interferes with osteopontin-mediated inhibition of apoptosis.

14. A method for determining whether an agent interferes with binding of osteopontin to CD44 receptor bearing cells, comprising:

(a) providing an osteopontin polypeptide;

(b) exposing said osteopontin polypeptide to CD44 receptor bearing cells in the presence of said agent; and

(c) measuring the binding of said osteopontin polypeptide to said CD44 receptor bearing cells;

whereby, binding of said osteopontin polypeptide to said CD44 receptor bearing cells in the presence of said agent that is less than the binding of said osteopontin polypeptide to said CD44 receptor bearing cells in the absence of said agent indicates that said agent interferes with binding of osteopontin to CD44 receptor bearing cells.

15. The method of claim 14 wherein said osteopontin polypeptide comprises a polypeptide having the sequence of SEQ ID NO: 1.

16. The method of claim 14 wherein said osteopontin polypeptide is mature human osteopontin.

17. The method of claim 14 wherein said CD44 receptor bearing cell is a metastatic tumor cell.

18. An isolated osteopontin polypeptide which binds to CD44, said osteopontin polypeptide comprising fewer than 100 amino acids.

19. The isolated osteopontin polypeptide of claim 14, said polypeptide comprising the osteopontin polypeptide of SEQ ID NO: 1.

20. The polypeptide of SEQ ID NO: 1.

21. An isolated polypetide fragment of the polypeptide of SEQ ID NO: 1 comprising at least 10 amino acids, said polypeptide selectively binding to CD44 receptor.

22. The isolated polypeptide fragment of claim 21 wherein said polypeptide comprises at least 15 amino acids.

23. The isolated polypeptide fragement of claim 22 wherein said polypeptide comprises at least 25 amino acids.

24. The isolated polypeptide fragement of claim 23 wherein said polypeptide comprises at least 50 amino acids.

25. An isolated polypeptide fragment of CD44, said isolated polypeptide fragment having osteopontin binding activity.

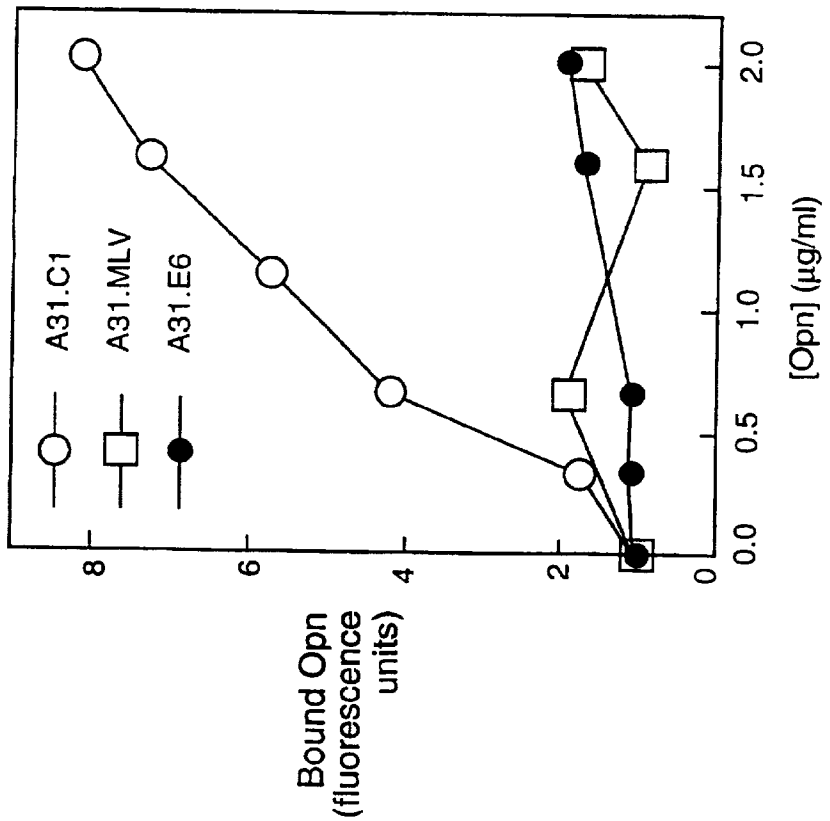

FIGURE 1A

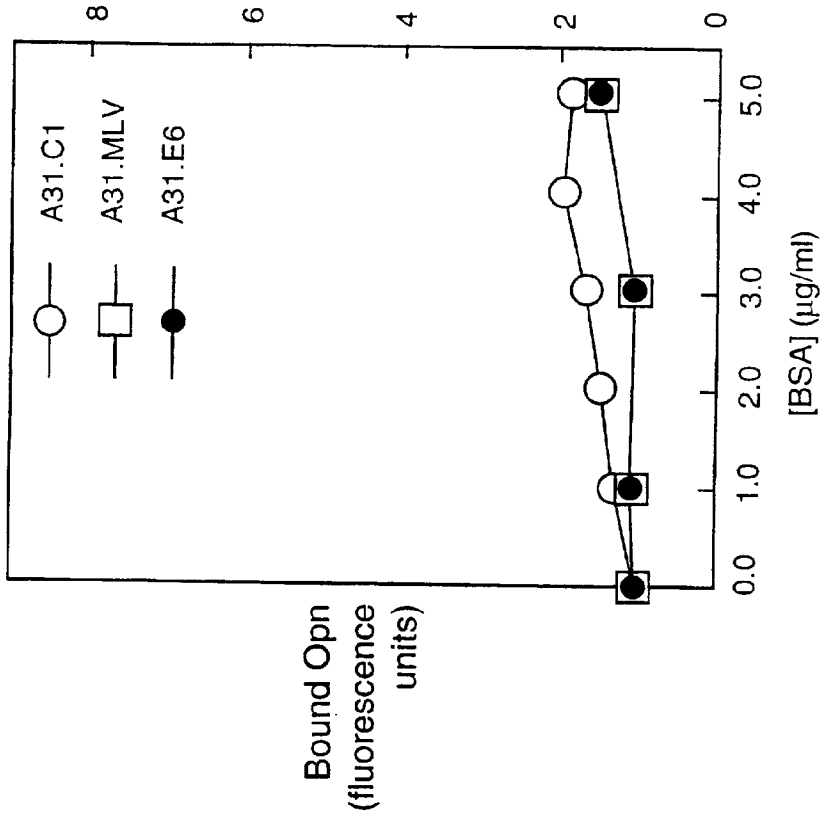

FIGURE 1B

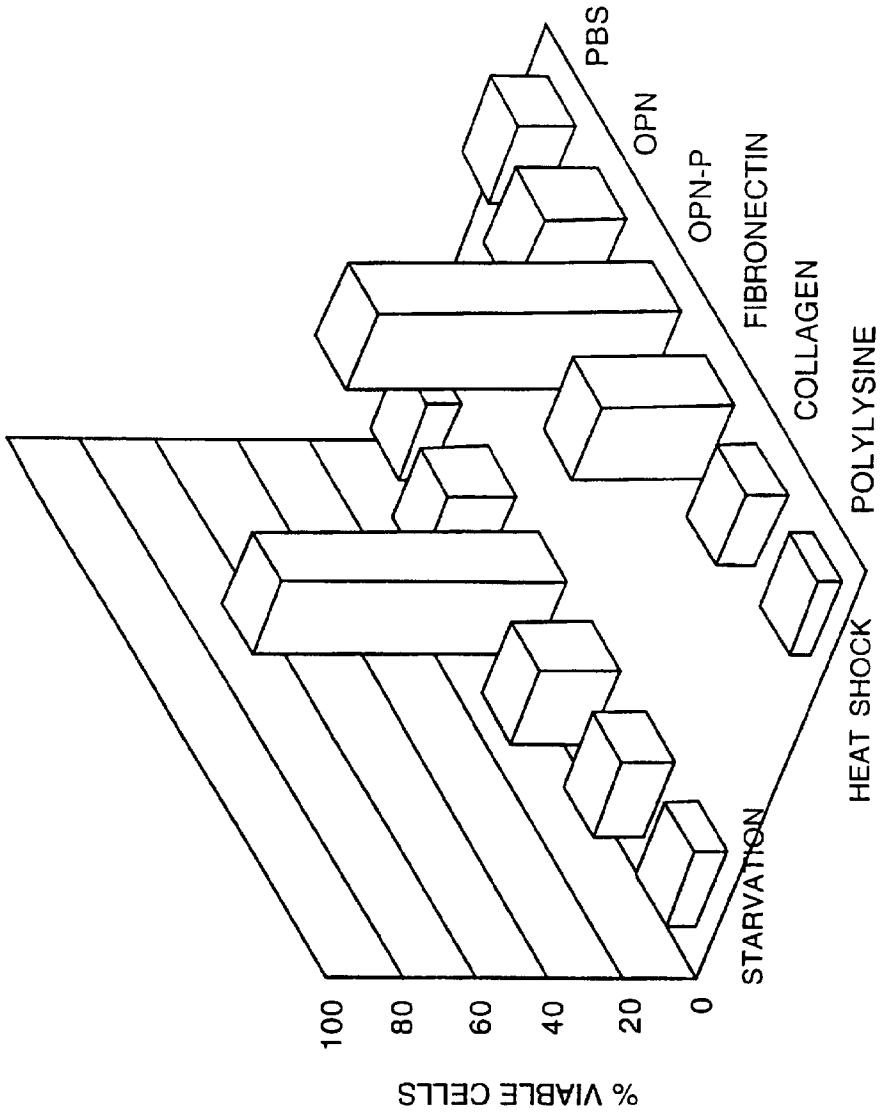
PBS
OPN
OPN-P
FIBRONECTIN
COLLAGEN
POLYLYSINE
HEAT SHOCK
STARVATION
100
80
60
40
20
0
% VIABLE CELLS

FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14153

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/2, 435/6, 435/7.2; 436/501; 530/324, 539/395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 6, 7.2; 436/ 501; 530/324, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, WPIDS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SENGER et al. Stimulation of endothelial Cell Migration by Vascular Permeability Factor/Vascular Endothelial Growth Factor through Cooperative Mechanisms Involving the $\alpha_v\beta_3$ Integrin, Osteopontin, and Thrombin. Amer. J. Path. July 1996, Vol. 149, No. 1, pages 293-305, see entire document.	1-25
Y	CHAMBERS, A. F. Regulation and Function of Osteopontin in Ras-Transformed Cells. Annals New York Acad. Sci. April 1995, Vol. 760, pages 101-108, see entire document.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 OCTOBER 1997	Date of mailing of the international search report 31 OCT 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer EVELYN RABIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14153

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YAMAMOTO et al. Effect of Recombinant Osteopontin on Adhesion and Migration of P388D1 Cells. Annals New York Acad. Sci. April 1995, Vol. 760, pages 378-380, see entire document.	1-25
Y	OATES et al. The Identification of Osteopontin as a Metastasis-Related Gene Product in a Rodent Mammary Tumour Model. Oncogene. July 1996, Vol. 13, pages 97-104, see entire document.	1-25
Y	DENHARDT et al. Overcoming Obstacles to Metastasis - Defenses Against Host Defenses: Osteopontin (OPN) as a Shield Against Attack by Cytotoxic Host Cells. J. Cell. Biochem. September 1994, Vol. 56, pages 48-51, see entire document.	1-25
Y	GUO et al. Identification of a ras-Activated Enhancer in the Mouse Osteopontin Promoter and Its Interaction with a Putative ETS-Related Transcription Factor Whose Activity Correlates with the Metastatic Potential of the Cell. Mol. Cell. Biol. January 1995, Vol. 15, No. 1, pages 476-487, see entire document.	1-25
Y	BAUTISTA et al. Inhibition of Arg-Gly-Asp (RGD)-Mediated Cell Adhesion To Osteopontin by a Monoclonal Antibody Against Osteopontin. J. Biol. Chem. 16 September 1994, Vol. 269, No. 37, pages 23280-23285, see entire document.	1-25
Y	BROWN et al. Osteopontin Expression and Distribution in Human Carcinomas. Amer. J. Path. September 1994, Vol. 145, No. 3, pages 610-623, see entire document.	1-25
Y	GARDNER, H. A. R. et al. Specific Reduction in Osteopontin Synthesis by Antisense RNA Inhibits the Tumorigenicity of Transformed Rat1 Fibroblasts. Oncogene. August 1994, Vol. 9, pages 2321-2326, see entire document.	1-25
Y	CHAMBERS et al. Adhesion of Metastatic, ras-Transformed NIH 3T3 Cells to Osteopontin, Fibronectin, and Laminin. Cancer Res. 01 February 1993, Vol. 53, pages 701-706, see entire document.	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14153

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 1/02; C12Q 1/68; G01N 33/53, 33/566, 33/567; A61K 38/00; C07K 1/00, 5/00, 7/00, 14/00, 16/00, 17/00